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Anticancer and Antioxidant Activity of the Greenly Synthesized Zinc Nanoparticles Composites using Aqueous Extract of *Withania Somnifera* plant

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Abstract

Withania somnifera L. is a medicinal herb related to Solanaceae family, known as ashwagandha. The aqueous extract of *W. somnifera* was utilized for the green synthesis of zinc nanoparticles to investigated their antioxidant and anticancer potentials. The synthesized zinc nanoparticle solution of *W. somnifera* extract was characterized by phytochemical analysis, Transmission Electron Microscope (TEM), and zeta potential. The results revealed reduced values of the phytochemical constitutes, large surface area, and high stability of the nanoparticles. The antioxidant activity of the extracted *W. somnifera* and its zinc nanoparticles was evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) assay, in which the original extract has the more antioxidant capacity with IC_{50}= 0.701 mg/mL, along with potent results for both samples relative to ascorbic acid. The extracted *W. somnifera*, its zinc nanoparticles, and zinc sulfate solution were *in vitro* assessed as anticancer agents on well-known six human tumor, and a normal cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. The results demonstrated that the synthesized zinc nanoparticles of the *W. somnifera* extract shown the most potent cytotoxicity with IC_{50}= 19.17 µg/mL on HeLa cell line. The synthesized nano-zinc solution of the *W. somnifera* aqueous extract performed to be proficient as a potent anticancer characteristic than the plant extract with developed biological potency.

Keywords: *Withania somnifera* L.; green synthesis; Phytochemical analysis; TEM; zeta potential; DPPH antioxidant, anticancer agents.

1. Introduction

Withania somnifera L. as a medicinal herb in Ayurveda is related to Solanaceae family known as ashwagandha. Commonly, it was applied for the treatments of musculoskeletal conditions, supplementary for energy, and benefit generally for human health especially for pregnant [1]. Various chemical and biological agents were generated in the course of the formation of non-specific increased resistance (SNIR) with a nonspecific mechanism, nevertheless counter numerous pathological parameters [2]. Cancer is a hyper-proliferative disorder, which led to restrained propagation, apoptosis dysregulation, and cell cycle. The extracted solutions of *W. somnifera* were found to be operative inhibitors for TCA cycle enzymes *i.e.* isocitrate dehydrogenase, malate dehydrogenase in colon cancer-bearing animals [3]. Withanolide steroidal lactones and Withaferin A (**Figure 1**) isolated from plant roots were reported to be potent anticancer for skin carcinoma in rats [4].

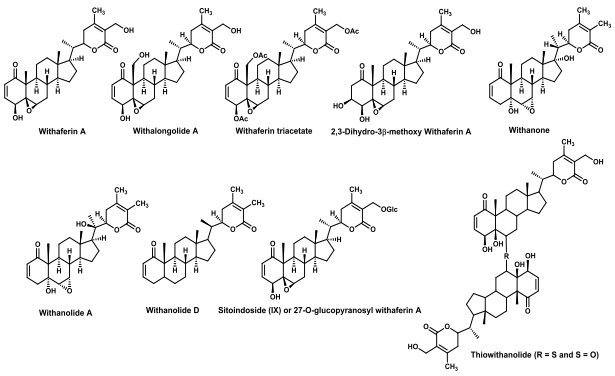

Figure 1: The structures of steroidal lactones isolated from leaves, roots, stem, and fruits of *W. somnifera*.

The anti-neoplastic activity was reported for *W. somnifera* Dunal on urethane that was induced lung-adenomas in adult male of albino mice [5]. The major chemical constituents of the extracted

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components were identified as Withaferin A, which activated mitochondrial apoptotic pathways, and induced the dysfunction of mitochondrial in human leukemia HL-60 cell lines [6]. Also, the extracts of *W. somnifera* displayed a potent anticancer profile on breast, pancreatic, prostate, renal, and fibrosarcoma cell lines [7-11]. *W. Somnifera* extract is considered one of the authoritative nutritional supplements through its containment of alkaloids components, and despite the classification of the plant extract as a toxic substance, it was found that it has an effective activity in combating the growth of the diverse of cancer cells according to the type of solvent used in the process of extracting the components of the plant from roots, leaves, stems, and fruits [12]. The use of whole plant extract in cancer therapy was interestingly investigated by increasing cell proliferation, white blood cells, and stem cell proliferation. The mechanism was based upon reactive oxygen species generated, and tumor cell proliferation. Halder *et al*. [13] have reported the anticancer potency of the root extract of *W. somnifera* on human malignant melanoma "A375" cell lines. Consequently, the whole plant alcoholic extract was applied as an anticancer agent on gastric, breast, colon [14], and alcoholic root extract on neuroblastoma cells [15], colon [16], murine B16F1 melanoma [17], cancer arise from natural estrogen receptor-negative mammary in MMTV/Neu Mice [18], and V79 Chinese hamster cells [19].

On the other side, the extracted *W. somnifera* particularly displayed privileged biological characteristics, hence these extracts of plant materials demonstrated hypoglycaemic [20, 21], antioxidant [22-24], anti-stress [25-28], anti-anxiety [29, 30], anti-aging [30], immunomodulator [31], antialzheimer, antiparkinsonian [32-36], anti-inflammatory [37-39], antimicrobial [40], anticancer [41-44], antiepileptic [45], cardioprotective [46], anticortisol [29], and antidepressant activities [47], and have macrophage-activating [48], and nootropic effects [49].

Herein, we report the preparation of *Withania somnifera* leaves aqueous extract and an eco-friendly protocol for the synthesis of zinc nano-solution using this plant extract to evaluate the biological characteristics of these samples as potent antioxidant, and anticancer agents with the hope to find alternative chemotherapeutic agents.

2. Materials and Methods

2.1. Plant material and extraction method

The leaves of *W. somnifera* as an herbal medicine were purchased from the roadsides in Iraq. The leaves were washed and dried at 45 ^{o}C in the oven for three days for perfect dryness. A weighed 20 gm of the beforehand dried leaves of the plant were settled in a conical flask, then deionized water (200 mL) was added. The conical flask was retained in a horizontal water bath shaker at 25 ^{o}C for two hours at 200 rpm. The prepared extract was filtered off using Whatman filter paper No. 1 (Whatman Int. Ltd., Kent, UK) using a Buchner funnel and stored at 5 ^{o}C for further analysis, after adjusting the final volume to 100 mL in volumetric flasks with the deionized water.

2.2. Reagents

Folin-Ciocalteu reagent (Fluka, Biochemical Inc., Bucharest, Romania), Gallic acid (Biomedical Inc., Orange City, FL, USA), 1,1-Diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$), $AlCl_3$, NaOH, $NaNO_2$, catechin, vanillin, hydrochloric acid, ascorbic acid, MTT, RPMI-1640 medium, and DMSO were obtained from Sigma Aldrich (St. Louis, USA). Na_2CO_3 (El-Nasr Pharmaceutical Chemicals, Cairo, Egypt). Fetal bovine serum (FBS; Gibco Life Technologies, Paisley, UK). Zinc sulfate (Andenex-Chemie, Hamburg, Germany). Doxorubicin was obtained from Merck (Darmstadt, Germany).

2.3. Synthesis of Metal Nanoparticles

A succeeding procedure was used for the preparation of the zinc nano-solution [50], prepared by the aqueous extract of *W. somnifera* leaves. Zinc sulfate solution was prepared in 1 mmol concentration in deionized water. Place 20 mL of the plant extract in a conical flask (500 mL), and add subsequently 20 mL of the salt solution with incessant stirring at pH= 7 (neutral medium) without the addition of puffer solution. The total volume of salt solution was completely added, stirring of the mixture was continued at 25 ^{o}C for two hours. Irradiation of the mixture was acquired by a special UV lamp with reduction influence possessions at a wavelength (λ = 254 nm) for 15 min, following the techniques reported by Sharma *et al*. [51], and Devasenan *et al.*, [52]. The prepared zinc nano-solution was assimilated in an equimolar ratio (1:1).

2.4. Instruments

The transmission electron microscopy measurements were analyzed on a (JEOL TEM-2100, Tokyo, Japan), and Zeta potential analysis was run on a "Malvern, Malvern Instruments Limited, Ver. 2.3, Grovewood Road, Malvern, Worcestershire, UK. WR14 1XZ" at the Electron Microscope Unit, Central Laboratory, Faculty of Agriculture, Mansoura University, Mansoura, Egypt. The horizontal water bath shaker (Memmert WB14, Schwabach, Germany), for the plant extraction process. Spectrophotometric apparatus (Spekol 11 spectrophotometer, analytic Jena AG, Jena, Germany). UV lamp (Vilber Lourmat-6.LC, VILBER Smart Imaging, Marne-la-Vallée, France).

2.5. Phytochemical analysis

The phytochemical analysis discovered a wide assortment of nutritive chemical constitutes, which have obvious effects on human health [53]. The tests were run to screen and classify the chemical categories of therapeutically active influence *i.e*. phenolic, flavonoid, and tannin contents of the anticipated *W. somnifera* leave aqueous extract and its zinc nanoparticles were accomplished to assess the relevance of the formed nanoparticles, interpretation of the metal nanoparticles by the amendment in phytochemical contents, and to disclose the representative bioactivity of the plant extract.

2.5.1. Total phenolic contents

The test was inspected for *W. somnifera* leaves aqueous extract and its zinc nanoparticles to calculate the phenolic contents. Folin-Ciocalteu (F-C) assay was utilized succeeding the method reported by Wolfe *et al* [54], and Issa *et al*. [55], in which a Gallic acid standard curve was applied to calculate the representative values as milligram Gallic acid equivalents/grams of the dried plant. The process concerned the use of the following equation of a Gallic acid standard curve ($y = 0.0062x$, $r^2 = 0.987$).

2.5.2. Total flavonoid contents

The contents of flavonoids are expressed as milligram catechin equivalent per gram of the dry weight of the plant. The test was inspected for *W. somnifera* leaves aqueous extract and its zinc nanoparticles. The test for this quantification was administered utilizing aluminum chloride colorimetric assessment succeeding the method reported by Zhishen *et al.* [56], applying the Catechin standard curve for the detection of secondary metabolite to calculate the total flavonoids from this equation ($y = 0.0028$ x, $r^2 = 0.988$).

2.5.3. Total tannin contents

The tannin contents were estimated by the method of vanillin-hydrochloride [57], in which the sample absorbance was measured after the treatment with freshly prepared vanillin-hydrochloride. The recorded values of tannin contents for *W. somnifera* leaves aqueous extract and its zinc nanoparticles, expressed as gram tannic acid equivalents / 100-gram dry plant, were calculated from tannic acid standard curve applying this equation ($y = 0.0009x$; $r^2 = 0.955$).

2.6. Potential Biological Characteristics

2.6.1. Antioxidant Activity Procedure

The antioxidant activity of *W. somnifera* leaves aqueous extract and its zinc nanoparticles was assessed by a colorimetric DPPH free radical assay [58]. The experimental procedure involved the preparation of a serial dilution of each sample "ten diluted bottles" from each tested sample using methanol for dilution step. Next, 1 mL of DPPH$^{\bullet}$ solution "0.135 mM" was added for each concentration of the sample in the serial dilution. Subsequently, the samples were kept in dark at room temperature for 30 minutes. The absorbance of the change in purple color of the samples was measured for each bottle at 517 nm. The % of remaining DPPH$^{\bullet}$ was calculated as followed: % Remaining DPPH$^{\bullet}$ = $[DPPH^{\bullet}]_T/[DPPH^{\bullet}]_{T=0} \times 100$

The IC_{50} values were calculated from a linear regression curve obtained from the exponential curve [59] by plotting the miscellaneous sample concentrations against the % of DPPH$^{\bullet}$ remaining. The % inhibition was calculated as followed: "% Inhibition = [(A control − A sample)/A control] × 100", in which "A control" is the absorbance of control, and "A sample" is the absorbance of each concentration.

2.6.2. Anticancer Activity Procedure

The human tumor, and normal cell lines were acquired from a holding company for biological products and vaccines (VACSERA), Cairo, Egypt. Six human tumor cells are defined as HePG-2 (Hepatocellular carcinoma), Hela (Epithelioid cervix carcinoma), HEP2 (Epidermoid larynx carcinoma), PC3 (Human prostate cancer), MCF-7 (Mammary

gland carcinoma), and HCT-116 (Colorectal carcinoma), and a normal lung fibroblast (WI-38) cell lines. Doxorubicin was selected as a potential anticancer standard. MTT assay was applied in this research as a colorimetric procedure to investigated the anticancer potency of the tested samples. Herein, we have applied the same method as reported by Bondock *et al*. [60]. The samples were prepared in a definite concentration "1.56-100 µg/mL" using RPMI-1640 medium for dilution process. DMSO was added for each sample with maximum ultimate concentration of 1% v/v. Culturing of cells was proceeded for ten days, then seeded in 96-well plates "1.0×10^4 cells/well" at 37ºC for 24 h under 5% carbon dioxide using a water-jacketed incubator. The medium was added, on the other side, without the serum for preparing the control sample, the cells were incubated, then addition of the medium to each sample concentration. Prepare a suspension of cells in RPMI-1640 medium, 1% penicillin, 1% streptomycin, and 1% L-glutamine at 37 ºC in a 5% CO_2 incubator. The plated were incubated for further 96 h, then the mixture was removed, plates were inverted onto a pad of paper towels, rinse the cells cautiously with medium, and fix it with formaldehyde (3.7%, v/v) in saline for 20 minutes. Again, rinse the cells with water for additional analysis. The reduction in color of the samples was measured at 570 nm using spectrophotometer apparatus. IC_{50} values were calculated from a straight linear regression using Origin 8.0® software (OriginLab Corporation). The values were calculated from a fit line *i.e*. Y= a*X + b, in which IC_{50}= (0.5-b)/a. The relative cell viability % was intended as "(A treated samples/A untreated sample) × 100".

3. Results and Discussions

The green protocols for the synthesis of metal nanoparticles from the extracts of natural plant sources, investigation of their change in the phytochemical contents, and characterization of the metal nanoparticles were recently reported [61-64].

3.1. Nanoparticles Characterization

3.1.1. Phytochemical investigation

The analysis of phytochemical constitutes was intended to determine the active constitutes of the *W. somnifera* leaves extract and its synthesized zinc nanoparticles. In this course, the analysis of this type quantitatively estimates the secondary metabolites of both samples, which were consumed in the generation of nanoparticles through a reduction process [65]. The phytochemical profile included the calculation of phenolic, flavonoid, and tannin contents to explore the supplementary factors that will increase the metal nanoparticles formation as these categorized groups act as reducing, and stabilizing agents in this green protocol of the synthesis. Hereafter, the results of the phytochemical analysis presented in Table 1 demonstrated that the aqueous *W. somnifera* leaves extract has rich phenolic contents (62.37 mg gallic acid equivalent / g dry extract), flavonoid contents (7.10 mg catechin equivalent / g dry extract), and tannin contents (11.21 mg gallic acid equivalent / g dry extract). The presence of phenolic groups provided an accessible opportunity for the reduction process of the metal ions into nanoparticles through electron resonance hybrid effects [66, 67]. Moreover, flavonoids, as phenolic subclass are considered as more stable chemical constitutes, could also contribute to the reduction process. The formed negative anions stabilized by electron resonance hybrid effects enable the reduction of the metal ions with a high degree resulting in the formation of metal ions in a zero-valent state that is related to the generated nanoparticles of smaller size. The process involved an increase in the surface area of the zinc nanoparticles, and accordingly improve the bioactivity of the zinc nanoparticles prepared by *W. somnifera* leaves extract [68]. The phytochemical analysis of zinc nanoparticles of *W. somnifera* leaves extract accessible a prominent decrease in the phenolic contents at (48.60 mg gallic acid equivalent / g dry extract) than the *W. somnifera* leaves extract. Also, a decrease in the flavonoid contents of zinc nanoparticles at 4.40 mg catechin equivalent / g dry extract. Similarly, the total tannins avowed the matching implication with 10.29 mg gallic acid equivalent / g dry extract for the solution of zinc nanoparticles.

3.1.2. Transmission Electron Microscope (TEM)

TEM scan was investigated to characterize the formation of zinc nanoparticles of *W. somnifera* leaves extract at a high spatial resolution (50 nm) (**Figure 2**). The performance was utilized to investigate the surface morphology of the metal

nanoparticles, as well as the shape, size, and aggregation of the generated nanoparticles. Otunola *et al.* [69], and Elsayed *et al.* [70], have stated the same sequence of nano-metal characterization to estimate the morphological properties of the nanoparticles.

Table 1: The data of the phytochemical analysis of *W. somnifera* leaves extract and its investigated zinc nanoparticles.

Samples	Phytochemical Analysis		
	Phenolic Contents [a]	Flavonoid Contents [b]	Tannin Contents [c]
W. somnifera extract	62.37	7.10	11.21
W. somnifera + ZnNPs	48.60	4.40	10.29

[a] mg gallic acid equivalent / g dry extract. [b] mg catechin equivalent / g dry extract. [c] mg gallic acid equivalent / g dry extract.

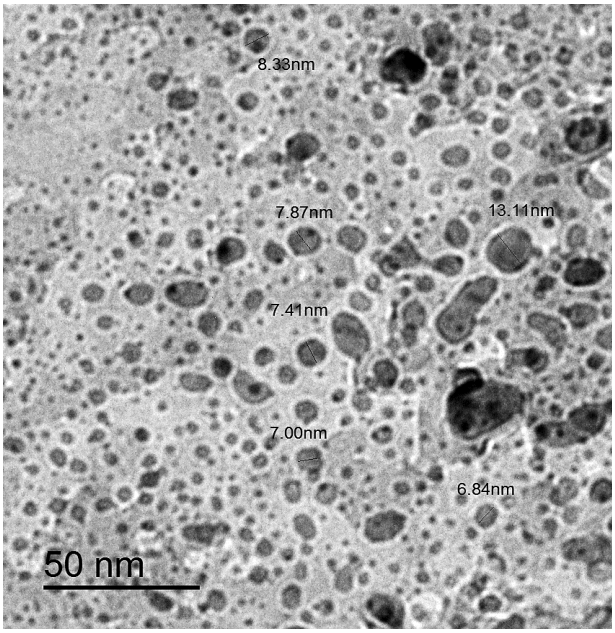

Figure 2: TEM screened micrographs of zinc nanoparticles prepared by *W. somnifera* extract at a magnification of 50 nm.

The morphological properties enable the study of the shape, size, and distribution of the nanoparticles. As it appeared from the TEM scan, the size of the nanoparticles is varied in the range of 6.84-13.14 nm with small sizes referring to enriched biological characters. The nanoparticles are more aggregated, hence an improvement in the biological potency is expected. The spherical shapes of the nanoparticles allowed a large surface area of the particles, and hence more efficiency than the original extract. The assessment of agglomeration and/or aggregation specified that the nanoparticles are frequently distributed in the scanned image (**Figure 2**). Hence, the generated nanoparticles of zinc prepared from *W. somnifera* leaves extract seems to be the efficient sample for potent cytotoxic effect than the *W. somnifera* leaves extract itself with a large surface area resulted from the spherical shapes, and aggregation of the nanoparticles.

3.1.3. Zeta potential

The analysis of zeta potential was applied to characterize the charges of the generated zinc nanoparticles by *W. somnifera* extract. The technique elaborated the aptitude of the charged nanoparticles to attract a thin layer of ions that have reverse charges with the charges of the nanoparticles. The stability of the nanoparticles in the solution was investigated through zeta potential magnitude. The stability range of this analysis is presented as zeta potential values within ±60 mV have exceptional stability, while zeta potential within ±10 mV directly resulted in a rapid agglomeration of the particles with the steric factor absence. The negative sign of zeta potential indicates that the value was recorded above the isoelectric point and the net charge of the scattered beam (**Figure 3**).

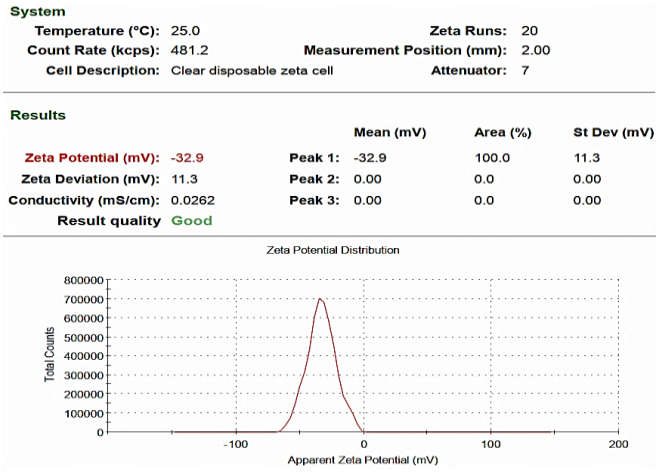

Figure 3: Zeta potential analysis for zinc nanoparticles synthesized by *W. somnifera* extract.

The sample was analyzed for zeta potential at Malvern Instruments Limited analysis with Ver.

2.3 (**Figure 3**) [71]. The range that zeta potential screening was recorded with ±100 mV. The results presented that zinc nanoparticles of *W. somnifera* extract has zeta potential at -32.9 mV, zeta deviation 11.3 mV, and conductivity 0.0262 mS/cm, in which exceptional stability of the zinc nanoparticles supplemented with zeta value in the range of ±60 mV as stated by Honary and Zahir [72].

Potential Antioxidant Activity

The antioxidant scavenging potency of the extracts of *Withania somnifera* L. leaves has extensively been investigated [22-24]. Therefore, the current work assessed the antioxidant activity of *W. somnifera* aqueous extract and its zinc nanoparticles solution calorimetrically by DPPH assay. The half-maximal inhibitory concentration refers to the calculated IC_{50} values that are the scavenged 50% of DPPH• radical by a definite sample concentration. The IC_{50} values were calculated from the exponential curves that are relations between the % of remaining DPPH and the sample concentration. Ascorbic acid was utilized as a reference standard for the appraisal scale with the obtained results of the tested samples. Table 2 presented the calculated IC_{50} values, % inhibitions, and % of remaining DPPH• radical at varied concentrations of the investigated samples. The results displayed that *W. somnifera* extract and its zinc nanoparticles have good antioxidant activities with IC_{50}= 0.701, and 0.794 mg/mL, respectively, comparative to ascorbic acid (IC_{50}= 0.0222 mg/mL).

The % inhibition of the samples at varying concentrations is in agreement with the IC_{50} values calculated for all samples. The % inhibition of *W. somnifera* extract was calculated as 52.113% at 0.79 mg/mL, as the most potent record for the antioxidant activity, while the highest % inhibition for the nano-solution was calculated as 64.366% at 1.16 0.79 mg/mL. To enable the comparison of the % inhibition of both samples, it was noticed that the *W. somnifera* extract and its zinc nanoparticles revealed % inhibition at 25.07, and 12.958%, respectively, at approximately a concentration of 0.1 mg/mL.

The results also indicated a slight decrease in the antioxidant potency of the synthesized nanoparticles solution as an indication for the formation of metal nanoparticles. The phytochemical contents are in a proportional relationship with the antioxidant efficiency. The mechanism of action of the antioxidant assay depends upon the formation of a sample-DPPH complex that stabilizes the free radicals of DPPH. The reactive oxygen species of the extract constitutes are the major factor that controls the chemical reaction with DPPH• radical. Thus, the extract of this plant is rich with steroidal lactones, *i.e*. Withaferin A, Withalongolide A, Withaferin triacetate, etc., which have reactive oxygen species that are eligible to form a sample-DPPH complex with a high percent to scavenging the free radicals. In the case of the synthesized zinc nanoparticles, the metal salt solution tended to form a complex with the ligands of the extract constitutes or supported the bio-reduction of metal ions into nanoparticles, which decrease the possibility to form a complex with DPPH radical [73-75, 86].

Table 2
The DPPH antioxidant results of *W. somnifera* aqueous extract and its zinc nanoparticles relative to the standard ascorbic acid.

Sample	Sample Concentration (mg/mL)	% Remaining DPPH	% Inhibition	IC_{50} (mg/mL)
W. somnifera extract	0.79	47.887	52.113	0.701
	0.395	60.0	40.0	
	0.1975	74.93	25.07	
	0.0988	85.211	14.789	
W. somnifera extract + ZnNPs	1.16	35.634	64.366	0.794
	0.5822	61.127	38.873	
	0.2911	80.141	19.859	
	0.1455	87.042	12.958	
Ascorbic acid	0.062	15.267	85.19	0.0222
	0.031	39.084	62.07	
	0.016	61.069	40.74	
	0.008	74.809	27.41	

Potential Anticancer Activity

The anticancer activity of aqueous extracts of *W. somnifera* leaves has extensively been investigated on breast, colon, liver, glioma, YKG1, glioblastoma, and neuroblastoma cell lines [76-79]. On the other hand, the aqueous extracts of *W. somnifera* leaves were recently reported to have high anticancer potency on hepatocellular carcinoma, sarcoma, breast, and glioma cell lines [80-82]. Recently, many studies have focused on studying metal nanoparticles of plant

extracts as potential bioactive agents [83-85]. In this work, the anticancer potency of *W. somnifera* aqueous extract and its zinc nanoparticles solution was *in vitro* evaluated by MTT assay. The method was a colorimetric technique used to measure the yellow color reduced to purple due to the generation of formazan through mitochondrial succinate dehydrogenases of living cell lines. The anticancer potency was investigated on miscellaneous human tumor cell lines such as HePG-2, MCF-7, HCT-116, PC3, HeP2, HeLa cancer cell lines, and normal WI-38 cell line. The results were in comparison with doxorubicin as a chemotherapeutic standard.

The IC_{50} values, referred to the concentration of the sample needed for growth inhibition of the cancer cells by 50%, were calculated in µg/mL. Correspondingly, an inverse proportional between the IC_{50} values and sample inhibitive potency. Therefore, the potent cytotoxicity is accompanied by lower IC_{50} values at lower sample concentrations. Table 3 presented the *in vitro* cytotoxic results of *W. somnifera* leaves aqueous extract and its zinc nanoparticles solution on the diverse tumor, and normal cell lines. The results demonstrated that the solution of the greenly synthesized zinc nanoparticles displayed more potent cytotoxicity than the *W. somnifera* leaves extract itself within IC_{50} = 19.17-44.78 µg/mL, along with non-cytotoxic effect on WI-38 normal cell line IC_{50} = 88.37 µg/mL. In due courses, the samples revealed non-cytotoxic effects on the normal WI-38 cell line with IC_{50} ranged from 76.99 to 92.41 µg/mL. Predominantly, the zinc nanoparticles solution of *W. somnifera* extract demonstrated potent cytotoxicity on HePG-2 (IC_{50}= 23.24 µg/mL), MCF-7 (IC_{50}= 31.96 µg/mL), HCT-116 (IC_{50}= 44.78 µg/mL), PC3 (IC_{50}= 40.52 µg/mL), HeP2 (IC_{50}= 27.31 µg/mL), and HeLa tumor cell line (IC_{50}= 19.17 µg/mL). These results are in strong to moderate cytotoxic effects when compared to the results of doxorubicin (IC_{50}= 4.18-8.89 µg/mL). It appears that both samples displayed good cytotoxic effects on all tumor cell lines with enhanced potency for zinc nanoparticles solution than the original alcoholic extract. The cytotoxicity of the samples depended on the nature of the tested cell line, the chemical composition of the inspected sample, and the surface area of the sample. Thus, the most obtained cytotoxicity was perceived for *W. somnifera* leaves aqueous extract and its zinc nanoparticles solution on HeLa cell line with IC_{50} = 32.11, and 19.17 µg/mL, respectively. Subsequently, *W. somnifera* extract showed the lowest cytotoxicity on PC3 cell line (IC_{50} = 61.24 µg/mL), while zinc nanoparticles solution of the *W. somnifera* aqueous extract displayed the lowest cytotoxicity on HCT-116 cell line (IC_{50} = 44.78 µg/mL). The cytotoxicity order of the synthesized zinc nano-solution of *W. somnifera* leaves extract on all the tested tumor cell lines to obey the following order: HeLa > HePG-2 > HeP2 > MCF-7 > PC3 > HCT-116 tumor cell lines.

The cytotoxic activity of zinc sulfate solution was inspected on all the tumor and normal cell lines to investigate their potency and to estimate the effect of this salt solution on the cytotoxic results obtained by the synthesized nanoparticles. In a sequence, zinc sulfate solution revealed moderate to non-cytotoxic effects on all the tumor cells with IC_{50} ranged from 34.62 to 62.86 µg/mL. The salt solution is a non-cytotoxic agent on the normal WI-38 cell line with IC_{50}= 91.08 µg/mL. The results are an indication of the role of the prepared nano-solution for cancer cells growth inhibition and for the nanoparticles generation. **Figure 4** specifies a comparison of the values of (IC_{50} in µg/mL) of *W. somnifera* leaves aqueous extract, its zinc nanoparticles, and zinc sulfate solution on all tumor cell lines relative to the anticancer standard.

Table 3
The calculated IC_{50} values of the investigated samples on the diverse human tumor, and normal cells.

Samples	***In vitro* Cytotoxicity, IC_{50} (µg/mL)** [a]						
	HePG-2	MCF-7	HCT-116	PC3	HeP2	HeLa	WI-38
Doxorubicin	4.55	4.18	5.25	8.89	8.57	5.59	76.99
W. somnifera extract	35.67	52.76	57.45	61.24	43.13	32.11	92.41
W. somnifera extract + ZnNPs	23.24	31.96	44.78	40.52	27.31	19.17	88.37
Zinc sulfate	36.75	53.89	59.26	62.86	44.1	34.62	91.08

[a] IC_{50} values in µg/mL: 1-10 µg/mL (very strong cytotoxicity), 11–20 µg/mL (strong cytotoxicity), 21–50 µg/mL (moderate cytotoxicity), 51–100 µg/mL (weak cytotoxicity) and above 100 µg/mL (non-cytotoxic effects).

The structure-activity relationships (SAR's) of the *W. somnifera* extract with the obtained results verified that the extract is a rich source with steroidal lactones, for instance, Withaferin A, Withalongolide A, Withaferin triacetate, etc., which are electron-rich sources that improve the cytotoxic results. On the other hand, the increased surface area of the prepared nano-solution is proficient with exceptional potency on all the investigated tumor cell lines.

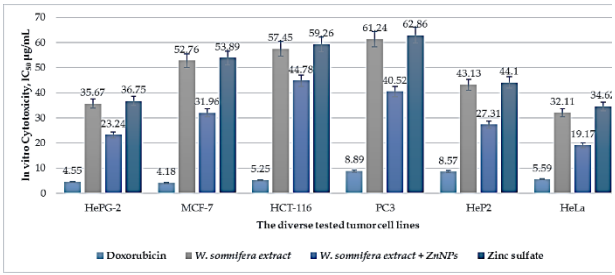

Figure 4: Comparison of the IC_{50} values of the inspected samples on the investigated tumor cells relative to doxorubicin.

Figure 5 characterizes the % inhibition calculated for *W. somnifera* leaves aqueous extract, its zinc nanoparticles, and zinc sulfate solution on all tumor cells comparative to the results of doxorubicin at seven serial dilutions (1.56-100 µg/mL). The highest concentration (100 µg/mL) demonstrated potent cytotoxicity than that of lower concentration (1.56 µg/mL). The values of % inhibition of the investigated compounds are matched with the calculated IC_{50} values. Therefore, the highest % inhibitions at 100 µg/mL were calculated for the aqueous extract of *W. somnifera* leaves, and its zinc nanoparticles with 38.506%, and 62.254, respectively, on Hela cell line. Zinc sulfate solution revealed the highest % inhibition on Hela cell line at 40.4% and the lowest % inhibition on WI-38 cell at 15.9%. The samples showed no cytotoxic potency at 1.56 µg/mL except for the prepared zinc nanoparticles that have very low cytotoxicity with % inhibition lower than 10 µg/mL. Generally, the cytotoxicity of the synthesized nanoparticles is better than the plant extract itself or zinc sulfate solution (*c.f.* **Figure 5** & **Table S1**). The cytotoxic potency depended upon the sample nature, chemical structure, and the tested cell line. Accordingly, the synthesized zinc nano-solution provided a large surface area of the particles, and more aggregated particles in nano-size, which is proficient for potent results.

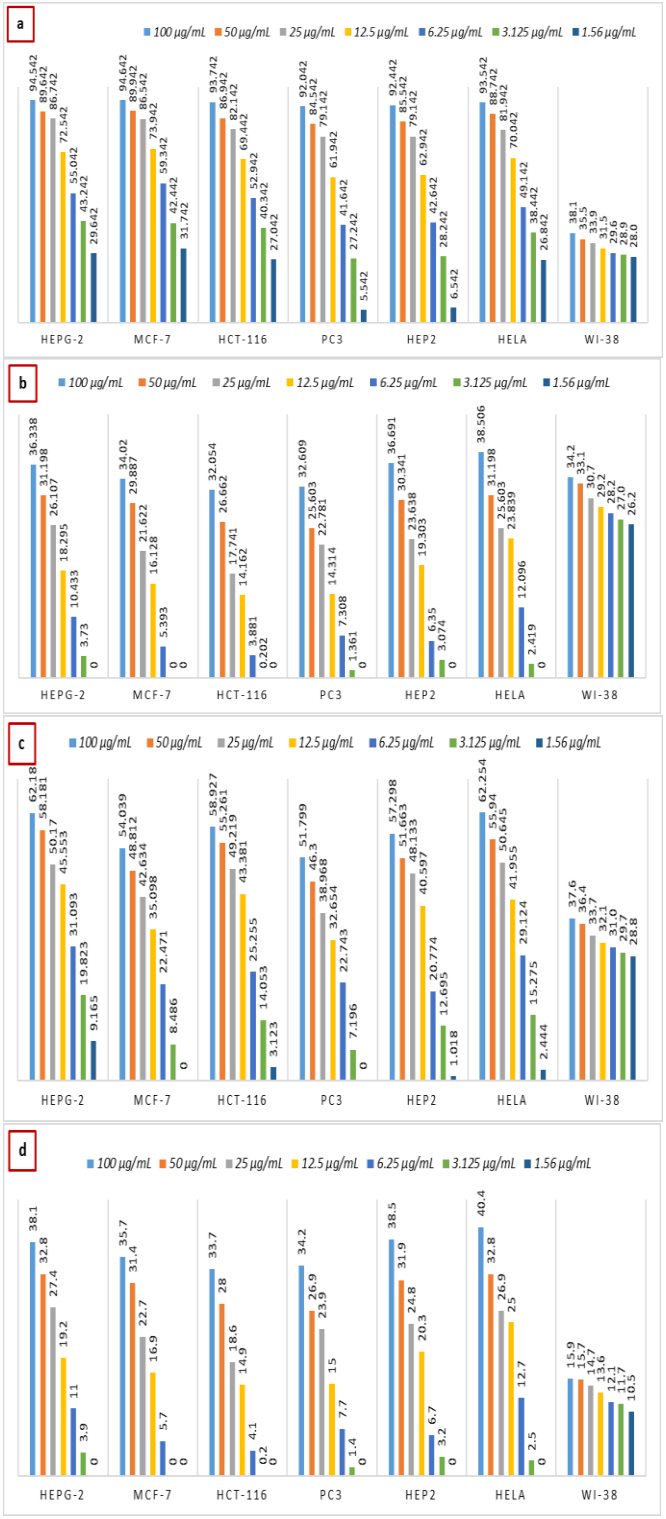

Figure 5: Comparison of the % inhibition of each sample against the diverse tumor, and normal cell lines at varied concentrations. **a**: Doxorubicin, **b**: *W. somnifera* Extract, **c**: *W. somnifera* + ZnNPs, **d**: Zinc sulfate.

Figure 6 indicated the representative average relative viability percentage of *W. somnifera* leaves aqueous extract, its zinc nanoparticles, zinc sulfate solution, and the standard doxorubicin on the human tumor, and normal cell lines at seven serial concentrations (1.56-100 µg/mL). The average relative viability percentage was recorded with high potency at the lower sample concentration (1.56 µg/mL). The results for the plant extract are varied from 63.662% on HePG-2, 65.98% on MCF-7, 67.946 on HCT-116, 67.391 on PC3, 63.309 on HeP2, 61.494 on Hela, and 65.8 on WI-38 cell lines, at the higher concentration (100 µg/mL). Furthermore, the nano-solution displayed improved cytotoxicity with a lower average relative viability percentage than the original plant extract on the tumor cell lines, along with comparable cytotoxicity on WI-38 cell. The results are in agreement with the calculated IC_{50} values, and % inhibitions at all concentrations (*c.f.* **Figure 6** & **Table S2**).

Supplementary Materials: Tables S1 & S2 are listed as supplementary sheets specified as: (Table S1. The % inhibition calculated at varied concentrations of the tested samples on the tumor, and normal cell lines). (Table S2. The percent of average relative viability at varied concentrations of the tested samples on all tumor, and normal cell lines).

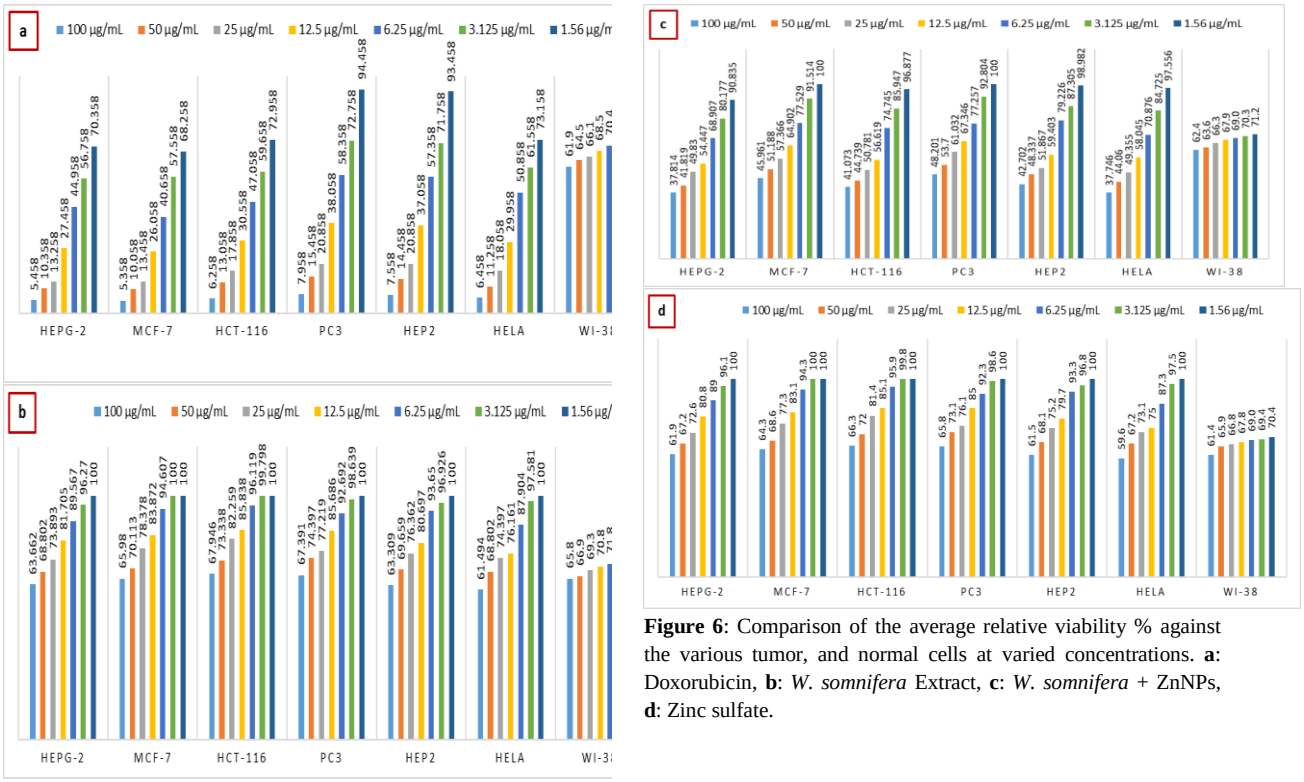

Figure 6: Comparison of the average relative viability % against the various tumor, and normal cells at varied concentrations. **a**: Doxorubicin, **b**: *W. somnifera* Extract, **c**: *W. somnifera* + ZnNPs, **d**: Zinc sulfate.

Table S1: The % inhibition calculated at varied concentrations of the tested samples on the tumor, and normal cell lines.

Samples	Conc. (µg/mL)	HePG-2	MCF-7	HCT-116	PC3	HeP2	Hela	WI-38
Doxorubicin	100	94.542	94.642	93.742	92.042	92.442	93.542	38.1
	50	89.642	89.942	86.942	84.542	85.542	88.742	35.5
	25	86.742	86.542	82.142	79.142	79.142	81.942	33.9
	12.5	72.542	73.942	69.442	61.942	62.942	70.042	31.5
	6.25	55.042	59.342	52.942	41.642	42.642	49.142	29.6
	3.125	43.242	42.442	40.342	27.242	28.242	38.442	28.9
	1.56	29.642	31.742	27.042	5.542	6.542	26.842	28.0
W. somnifera Extract	100	36.338	34.02	32.054	32.609	36.691	38.506	34.2
	50	31.198	29.887	26.662	25.603	30.341	31.198	33.1
	25	26.107	21.622	17.741	22.781	23.638	25.603	30.7
	12.5	18.295	16.128	14.162	14.314	19.303	23.839	29.2

	6.25	10.433	5.393	3.881	7.308	6.35	12.096	28.2
	3.125	3.73	0	0.202	1.361	3.074	2.419	27.0
	1.56	0	0	0	0	0	0	26.2
W. somnifera + ZnNPs	100	62.186	54.039	58.927	51.799	57.298	62.254	37.6
	50	58.181	48.812	55.261	46.3	51.663	55.94	36.4
	25	50.17	42.634	49.219	38.968	48.133	50.645	33.7
	12.5	45.553	35.098	43.381	32.654	40.597	41.955	32.1
	6.25	31.093	22.471	25.255	22.743	20.774	29.124	31.0
	3.125	19.823	8.486	14.053	7.196	12.695	15.275	29.7
	1.56	9.165	0	3.123	0	1.018	2.444	28.8
Zinc sulfate	100	38.1	35.7	33.7	34.2	38.5	40.4	15.9
	50	32.8	31.4	28	26.9	31.9	32.8	15.7
	25	27.4	22.7	18.6	23.9	24.8	26.9	14.7
	12.5	19.2	16.9	14.9	15	20.3	25	13.6
	6.25	11	5.7	4.1	7.7	6.7	12.7	12.1
	3.125	3.9	0	0.2	1.4	3.2	2.5	11.7
	1.56	0	0	0	0	0	0	10.5

Table S2

The percent of average relative viability at varied concentrations of the tested samples on all tumor, and normal cell lines.

Samples	Conc. (µg/ mL)	HePG-2	MCF-7	HCT-116	PC3	HeP2	Hela	WI-38
Doxorubicin	100	5.458	5.358	6.258	7.958	7.558	6.458	61.9
	50	10.358	10.058	13.058	15.458	14.458	11.258	64.5
	25	13.258	13.458	17.858	20.858	20.858	18.058	66.1
	12.5	27.458	26.058	30.558	38.058	37.058	29.958	68.5
	6.25	44.958	40.658	47.058	58.358	57.358	50.858	70.4
	3.125	56.758	57.558	59.658	72.758	71.758	61.558	71.1
	1.56	70.358	68.258	72.958	94.458	93.458	73.158	72.0
W. somnifera Extract	100	63.662	65.98	67.946	67.391	63.309	61.494	65.8
	50	68.802	70.113	73.338	74.397	69.659	68.802	66.9
	25	73.893	78.378	82.259	77.219	76.362	74.397	69.3
	12.5	81.705	83.872	85.838	85.686	80.697	76.161	70.8
	6.25	89.567	94.607	96.119	92.692	93.65	87.904	71.8
	3.125	96.27	100	99.798	98.639	96.926	97.581	73.0
	1.56	100	100	100	100	100	100	73.8
W. somnifera + ZnNPs	100	37.814	45.961	41.073	48.201	42.702	37.746	62.4
	50	41.819	51.188	44.739	53.7	48.337	44.06	63.6
	25	49.83	57.366	50.781	61.032	51.867	49.355	66.3
	12.5	54.447	64.902	56.619	67.346	59.403	58.045	67.9
	6.25	68.907	77.529	74.745	77.257	79.226	70.876	69.0
	3.125	80.177	91.514	85.947	92.804	87.305	84.725	70.3
	1.56	90.835	100	96.877	100	98.982	97.556	71.2
Zinc sulfate	100	61.9	64.3	66.3	65.8	61.5	59.6	61.4
	50	67.2	68.6	72	73.1	68.1	67.2	65.9
	25	72.6	77.3	81.4	76.1	75.2	73.1	66.8
	12.5	80.8	83.1	85.1	85	79.7	75	67.8
	6.25	89	94.3	95.9	92.3	93.3	87.3	69.0
	3.125	96.1	100	99.8	98.6	96.8	97.5	69.4
	1.56	100	100	100	100	100	100	70.4

4. **Conclusions**

The present study investigated an aqueous extraction of the *W. somnifera* leaves and utilized this extract for a green synthetic protocol for the preparation of zinc nanoparticles. The prepared zinc nanoparticles were broadly characterized by quantitative phytochemical analysis, TEM, and zeta potential. The phytochemical profile is notable with a decrease in the chemical constitutes in the nano-solution that

were consumed in the reduction process of the metal ions into a zero-valent state of the obtained nanoparticles. TEM scan estimated that the nanoparticles are in a small size, spherical shapes, and more aggregation. The zeta potential analysis demonstrated a negative sign of zeta potential value, with high stability of the produced nanoparticles. The samples also displayed good antioxidant scavenging capacity compared to ascorbic acid with a slight decrease in the antioxidant activity of the nano-solution prepared from the *W. somnifera* leaves aqueous extract. The consumption of phytochemical constitutes that are reactive oxygen species in the reduction process for the synthesis of zinc nanoparticles. Additionally, the anticancer activity of the samples was evaluated on six human tumor cells, and a WI-38 cell using MTT assay. The samples revealed moderate to non-cytotoxic effects on the normal cell line, along with potent cytotoxicity on six human tumor cells. In general, the synthesized zinc nanoparticles of *W. somnifera* leaves aqueous extract displayed the most potent cytotoxicity than the original aqueous extract of the plant on all the tumor cell lines. The nano-size, along with aggregation, the surface sign, and stability of the nanoparticle is the factor that controls the cytotoxic mechanism. Also, the results of cytotoxicity are related to the type of tumor cell line, and the chemical constitutes of each sample. Therefore, due to the satisfactory biological efficiency of the *W. somnifera* leaves aqueous extract, and its zinc nanoparticles, their therapeutic perspective is highly appreciated to pharmaceutical industries, and drug design, as well as food processing.

5. Conflicts of interest

"There are no conflicts to declare".

6. Formatting of funding sources

Self

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8. References

[1]. Jana, S. N.; Charan, S. M. Health Benefits and Medicinal Potency of *Withania somnifera*: A Review. Int. J. Pharm. Sci. Rev. Res., 2018, 48(1), Article No. 08, 22-29.

[2]. Singh, N.; Singh, S.P.; Sinha, J.N.; Shanker, K.; Kohli, R.P. *Withania somnifera* (Ashwagandha) A rejuvenator herbal drug which enhances survival during stress (An adaptogen). Int. J. Crude Res. 1982, 3, 29–35.

[3]. Muralikrishnan, G.; Dinda, A.K.; Shakeel, F. Immunomodulatory effects of *Withania somnifera* on azoxymethane induced experimental colon cancer in mice. Immunol Invest. 2010, 39, 688–98. doi: 10.3109/08820139.2010.487083

[4]. Mathur, S.; Kaur, P.; Sharma, M.; Katyal, A.; Singh, B.; Tiwari, M. *et al*. The treatment of skin carcinoma, induced by UV B radiation, using 1-oxo-5-beta, 6beta-epoxy-witha-2-enolide, isolated from the roots of *Withania somnifera*, in a rat model. Phytomedicine, 2004, 11, 452–460.

[5]. Singh N, Singh SP, Nath R, Singh DR, Gupta ML, Kohli RP, *et al*. Prevention of urethane induced Lung adenomas by *Withania somnifera* (L) Dunal in albino mice. Int. J. Crude Res. 1986, 24, 90–100.

[6]. Malik, F.; Kumar, A.; Bhushan, S.; Khan, S.; Bhatia, A.; Suri, K.A. *et al*. Reactive oxygen species generation and mitochondrial dysfunction in the apoptotic cell death of human myeloid leukemia HL-60 cells by a dietary compound withaferin A with concominant protection by *N*-acetyl cysteine. Apoptosis 2007, 12, 2115–2133.

[7]. Jayaprakasam, B.; Zhang, Y.; Seeram, N.P.; Nair, M.G. Growth inhibition of human tumor cell lines by withanolides from *Withania somnifera* leaves. Life Sci. 2003, 74, 125–132.

[8]. Yu, Y.; Hamza, A.; Zhang, T.; Gu, M.; Zou, P.; Newman, B. *et al*. Withaferin A targets heat shock protein 90 in pancreatic cancer cells. Biochem. Pharmacol. 2010, 79, 542–551. doi: 10.1016/j.bcp.2009.09.017.

[9]. Yadav, B.; Bajaj, A. Saxena M, Saxena AK. In Vitro Anticancer Activity of the Root, Stem and Leaves of Withania somnifera against Various Human Cancer Cell Lines. Indian J. Pharm. Sci. 2010, 72, 659–663. doi: 10.4103/0250-474X.78543

[10]. Yang, E.S.; Choi, M.J.; Kim, J.H.; Choi, K.S.; Kwon, T.K. Withaferin A enhances radiation-induced apoptosis in Caki cells through induction of reactive oxygen species, Bcl-2 downregulation and Akt inhibition. Chem. Biol. Interact. 2011, 190, 9–15. doi: 10.1016/j.cbi.2011.01.015.

[11]. Prakash, J.; Gupta, S.K.; Dinda, A.K. *Withania somnifera* root extract prevents DMBA-induced squamous cell carcinoma of skin in Swiss albino mice. Nutr. Cancer. 2002, 42, 91–97.

[12]. Dutta, R.; Khalil, R.; Green, R.; Mohapatra, S. S.; Mohapatra, S. *Withania Somnifera* (Ashwagandha) and Withaferin A: Potential in Integrative Oncology. Int. J. Mol. Sci. 2019, 20, 5310; doi:10.3390/ijms20215310

[13]. Halder, B.; Singh, S.; Thakur, S. S. *Withania somnifera* Root Extract Has Potent Cytotoxic Effect against Human Malignant Melanoma Cells. PLoS ONE 2015, 10(9), e0137498. doi:10.1371/journal.pone.0137498

[14]. Mulabagal, V.; Subbaraju, G.V.; Rao, C.V.; Sivaramakrishna, C.; Dewitt, D.L.; Holmes, D.; Sung, B.; Aggarwal, B.B.; Tsay, H.S.; Nair, M.G. Withanolide sulfoxide from Aswagandha roots inhibits nuclear

transcription Factor-Kappa-B, cyclooxygenase and tumor cell proliferation. Phytother. Res. 2009, 23, 987–992.

[15]. Caputi, F.F.; Acquas, E.; Kasture, S.; Ruiu, S.; Candeletti, S.; Romualdi, P. The standardized *Withania somnifera* Dunal root extract alters basal and morphine-induced opioid receptor gene expression changes in neuroblastoma cells. BMC Complement. Altern. Med. 2018, 18, 9.

[16]. Henley, A.B.; Yang, L.; Chuang, K.L.; Sahuri-Arisoylu, M.; Wu, L.H.; Bligh, S.W.; Bell, J.D. *Withania somnifera* Root Extract Enhances Chemotherapy through 'Priming'. PLoS ONE 2017, 12, e0170917.

[17]. Sudeep, H.V.; Gouthamchandra, K.; Venkatesh, B.J.; Prasad, K.S. Viwithan, a Standardized *Withania somnifera* Root Extract Induces Apoptosis in Murine Melanoma Cells. Pharmacogn. Mag. 2018, 13, S801–S806.

[18]. Khazal, K.F.; Hill, D.L.; Grubbs, C.J. Effect of *Withania somnifera* root extract on spontaneous estrogen receptor-negative mammary cancer in MMTV/Neu mice. Anticancer Res. 2014, 34, 6327–6332.

[19]. Turrini, E.; Calcabrini, C.; Sestili, P.; Catanzaro, E.; De Gianni, E.; Diaz, A.R.; Hrelia, P.; Tacchini, M.; Guerrini, A.; Canonico, B.; *et al*. *Withania somnifera* Induces Cytotoxic and Cytostatic Effects on Human T Leukemia Cells. Toxins (Basel) 2016, 8, 147.

[20]. Kumar, V.; Dey, A. Chatterjee Sunder S, Phytopharmacology of Ashwagandha as an antidiabetic herb, Springer International Publishing, 2017, 3, 37-68.

[21]. Teli, N.; Bagwe, T.; Kandampully, A.; Pala, B. *Withania somnifera* (Ashwagandha): A source of therapeutic agents, Int. J. Curr. Res. Chem. Pharm. Sci., 2014, 1, 36-43.

[22]. Uttara, B.; Singh, V.A.; Zamboni, P.; Mahajan, T.R. Oxidative stress and neurodegenerative diseases: A review of upstream and downstream antioxidant therapeutic options, Curr. Neuropharmacol. 2009, 7(1), 65-74.

[23]. Bhattacharya, S.K.; Satyan, K.S.; Ghosal, S. Antioxidant activity of glycowithanolides from *Withania somnifera*, Indian J. Exp. Biol. 1997, 35(3), 236-239.

[24]. Sumathi, S.; Padma, P.R.; Gathampari, S.; Vidhya, S. Free radical scavenging activity of different parts of *Withania somnifera*, Ancient Science of Life, XXVI 2007, (3), 30-34.

[25]. Verma, S. Pharmacological activity of *Withania somnifera*. World J. Pharm. Pharm. Sci., 2016, 5, 602-605.

[26]. Ashok Amit, G.; Shende, M.B.; Chothe, D.S. Antistress activity of Ashwagandha (*Withania somnifera* Dunal)-A review, Int. Ayurvedic Med. J. 2014, 2(3), 386- 393.

[27]. Trivedi Bihari, A.; Mahajan, D. Ashwagandha-The powerful antistress herb, World J. Pharm. Life Sci., 2017, 3, 177-180.

[28]. Singh, N.; Verma, P.; Pandey, R.B.; Gilca, M. Role of *Withania somnifera* and treatment of cancer: An overview, Int. J. Pharm. Sci. Drug Res. 2011, 3(4), 274-279.

[29]. Chandrasekhar, K.; Kapoor, J.; Anishetty, S. A prospective, randomized double blind, placebo controlled study of safety efficacy of a high concentration full-spectrum extract of Ashwagandha root in reducing stress and anxiety in adults, Indian J Psychol Med, 2012, 34(3), 274-279.

[30]. Singh, G.; Sharma, P.K.; Dudhe, R.; Singh, S. Biological activities of *Withania somnifera*, Ann. Biol. Res. 2010, 1(3), 56-63.

[31]. Ziauddin, M.; Phansalkar, N.; Patki, P.; Diwanay, S.; Patwardhan, B. Studies on the immunomodulatory effects of Ashwagandha, J. Ethnopharmacol. 1996, 50(2), 69-76.

[32]. Kuboyama, K.; Thoda, C.; Komatsu, K. Effects of Ashwagandha (Roots of *Withania somnifera*) on neurodegenerative diseases, Biol. Pharm. Bull. 2014, 37(6), 892-897.

[33]. Ahmad, M.; Saleem, S.; Ahmad, A.S.; Ansari, M.A.; Yousuf, S.; Hoda, M.N. Neuroprotective effects of *Withania somnifera* on 6- hydroxydopamine induced Parkinsonism in rats, Hum. Exp. Toxicol. 2005, 24(3), 137-147.

[34]. Kumar, A.; Kulkarni, S.K. Effect of BR-16A (Mentat) a polyherbal formulation on drug-induced catalepsy in mice, Indian J. Exp. Biol. 2016, 44(1), 45-48.

[35]. Gupta Lal, G.; Rana, A.C. Plant review *Withania somnifera* (Ashwagandha): A review, Pharmacogn. Rev., 2017, 1, 129-136.

[36]. Majumder, B. Role of protein vibration in antialzheimer's effect of Ashwagandha (*Withania somnifera*)-An analytical approach. Int. J. Biophys., 2017, 7(3), 41- 47.

[37]. Khan, M.A.; Subramaneyaan, M.; Arora, V.K.; Banerjee, B.D.; Ahmed, R.S. Effect of *Withania somnifera* (Ashwagandha) root extract on amelioration of oxidative stress and autoantibodies production in collagen-induced arthritic rats. J. Complement. Inteqr. Med. 2015, 12(2), 117-125.

[38]. Giri, R.K. Comparative study of anti-inflammatory activity of *Withania somnifera* (Ashwagandha) with hydrocortisone in experimental animals (Albino rats). J. Med. Plants Stud. 2016, 4(1), 78-83.

[39]. Singh, A.; Malhotra, S.; Subban, R. Anti-inflammatory and analgesic agents from Indian medicinal plants. Int. J. Integr. Biol. 2008, 3, 57-72.

[40]. Meher Kumar, S.; Das, B.; Panda, P.; Bhuyan, G.C.; Rao, M.M. Uses of *Withania somnifera* (Linn) Dunal (Ashwagandha) in ayurveda and its pharmacological evidences. Res. J. Pharmacol. Pharmacodyn. 2016, 8, 131-135.

[41]. Ahmed, W.A.; Mohamed, A.; Nasser, E. A., *et al*. Potential toxicity of Egyptian Ashwagandha: Significance for their therapeutic bioactivity and anticancer properties. Int. J. Sci. Res. 2014, 4(2), 2170-2176.

[42]. Ahmed, R.; Fatima, N.; Srivastava, A.N.; Khan, M.A. Anticancer potential of medicinal plants *Withania somnifera*, *Tinospora cordifolia* and *Curcuma longa*: A review. J. Medicinal Aromat. Plants 2015, 3, 47-56.

[43]. McKenna, M.K.; Gachuki, B.W.; Alhakeem, S.S.; Oben, K.N.; Rangnekar, V.M.; Gupta, R.C.; Bondada, S. Anticancer activity of Withaferin A in B-cell lymphoma. Cancer Biol. Ther. 2015, 16(7), 1088-1098.

[44]. Yadav, B.; Bajaj, A.; Saxena, M.; Saxena, A.K. *In vitro* anticancer activity of the root, stem and leaves of *Withania somnifera* against various human cancer cell lines. Indian J. Pharm. Sci. 2010, 72(5), 659-663.

[45]. Kulkarni, S.K.; Sharma, A.; Verma, A.; Ticku, M.K. GABA receptor mediated anticonvulsant action of

Withania somnifera root extract. Indian Drugs 1993, 305-312.

[46]. Mohanty, I.; Arya Singh, D.; Dinda, A.; Talwar Kishan, K.; Joshi, S.; Gupta Kumar, S. Mechanisms of cardioprotective effect of *Withania somnifera* in experimentally induced Myocardial Infarction. Basic Clin Pharmacol Toxicol. 2004, 94, 184-190.

[47]. Bhattacharya, S.K.; Bhattacharya, A.; Sairam, K.; Ghosal, S. Anxiolytic-antidepressant activity of *Withania somnifera* glycowithanolides: an experimental study. Phytomedicine 2000, 7(6), 463-469.

[48]. Devi, P.U. *Withania somnifera* dunal (Ashwagandha): Potential plant source of a promising drug for cancer chemotherapy and radiosensitisation. Ind. J.Exp. Biol. 1999, 34(10), 927-932.

[49]. Zhao, J.; Nakamura, N.; Hattori, M.; Kuboyama, T.; Thoda, C.; Komatsu, K. Withanolide derivatives from the roots of *Withania somnifera* and their neurite outgrowth activities. Chem. Pharm. Bull. Tokyo, 2002, 50(6), 760-765.

[50]. El-Refai, A.A.; Ghoniem, G.A.; El-Khateeb, A.Y.; Hasaan, M.M. Eco-friendly synthesis of metal nanoparticles using ginger and garlic extracts as biocompatible novel antioxidant and antimicrobial agents. J. Nanostruct. Chem. 2018, *8*, 71–81, doi:10.1007/s40097-018-0255-8.

[51]. Sharma, R.; Tahiliani, S.; Jain, N.; Priyadarshi, R.; Chhangani, S.; Purohit, S.; Joshi, P. Cynodon dactylon leaf extract assisted green synthesis of silver nanoparticles and their anti-microbial activity. Adv. Sci. Eng. Med. 2013, 5, 858–863.

[52]. Devasenan, S.; Beevi, N.H.; Jayanthi, S.S. Green synthesis and characterization of zinc nanoparticle using *Andrographis paniculata* leaf extract. Int. J. Pharm. Sci. Rev. Res. 2016, *39*, 243–247.

[53]. Youssef HA, Ali SM, Sanad MI, Dawood DH. Chemical Investigation of Flavonoid, Phenolic acids Composition and Antioxidant activity of Tagetes erecta Flowers. Egypt. J. Chem. 2020; 63(7), 2605-2615.

[54]. Wolfe, K.; Wu, X.; Liu, R. Antioxidant activity of apple peels. J. Agric. Food Chem. 2003, 51, 609–614.

[55]. Issa, N.K.; Abdul Jabar, R.S.; Hammo, Y.H.; Kamal, I.M. Antioxidant activity of apple peels bioactive molecules extractives. Sci. Technol. 2020, 10, 76–88. doi:10.5923/j.scit.20160603.03.

[56]. Zhishen, J.; Mengcheng, T.; Jianming, W. Research on antioxidant activity of flavonoids from natural materials. Food Chem. 1999, *64*, 555–559.

[57]. Burlingame, B. Wild nutrition. J. Food Compost. Anal. 2000, 13, 99–100, doi:10.1006/jfca.2000.0897.

[58]. Kitts, D.; Wijewickreme, A.; Hu, C. Antioxidant properties of a1 North American ginseng extract. Mol. Cell. Biochem. 2000, 203, 1–10.

[59]. Suciyati, S.; Adnyana, I. K.; Sukrasno, S.; Kurniatiorcid, N. F., Antioxidant and Anti-Inflammatory Activity of Red Ginger (Zingiber officinale Roscoe var. Sunti Val) Essential Oil Distillation Residues. Egypt. J. Chem. 2021 (in Press) doi: 10.21608/ejchem.2021.52477.3083

[60]. Bondock, S.; Adel, S.; Etman, H.A.; Badria, F.A. Synthesis and antitumor evaluation of some new 1,3,4-oxadiazole-based heterocycles. Eur. J. Med. Chem. 2012, 48, 192–199.

[61]. Farhan SA, Dadoosh RM, Jassim A. Evaluation of Phytochemical, Total phenolic and Antioxidant Activity of Carica Papaya Seed for Its Use in Biosynthesis of Gold Nanoparticles. Egypt. J. Chem. Articles in Press, Accepted Manuscript, Available Online 19 April 2021.

[62]. El-Shahaby, O.; Reicha, F.M.; Aboushadi, M.M.; El-Zayat, M. Green Synthesis and Biological Assessments of Silver Nanoparticles Using the Plant Extract of *Crataegus sinaica* Boiss. Fruits. J. Prog. Chem. Biochem. Res. 2020, 3 (2), 105-113, doi: 10.33945/SAMI/PCBR.2020.2.3.

[63]. El-Shahaby, O.A.; El-Zayat, M.M.; Rabei, R.; Aldesuquy, H.S. Phytochemical constituents, antioxidant activity and antimicrobial potential of *Pulicaria incisa* (lam.) DC as a folk medicinal plant. Prog. Chem. Biochem. Res. 2019, 2, 222–227, doi:10.33945/SAMI/PCBR.2019.4.7.

[64]. El-Zayat, M. M.; Eraqi, M. M.; Alrefai, H.; El-Khateeb, A.Y.; Ibrahim, M. A.; Aljohani, H. M.; Aljohani, M. M.; Elshaer, M. M. The Antimicrobial, Antioxidant, and Anticancer Activity of Greenly Synthesized Selenium and Zinc Composite Nanoparticles Using *Ephedra aphylla* Extract. Biomolecules 2021, 11, 470. https://doi.org/10.3390/biom11030470

[65]. Mohamed, HY.G.; Ismael, E.; Elaasser, M M.; Khalil, M M.H., Green Synthesis of Zinc Oxide Nanoparticles Using Portulaca oleracea (Regla Seeds) Extract and Its Biomedical Applications. Egypt. J. Chem. 2021, 64 (2), 661-672. doi: 10.21608/ejchem.2020.45592.2930

[66]. Ghosh, S.; Patil, S.; Ahire, M.; Kitture, R.; Kale, S.; Pardesi, K.; Cameotra, S.S.; Bellare, J.; Dhavale, D.D.; Jabgunde, A.; *et al*. Synthesis of silver nanoparticles using *Dioscorea bulbifera* tuber extract and evalution of its synergistic potential in combination with antimicrobial agents. Int. J. Nanomed. 2012, 7, 483–496.

[67]. Egorova, E.M.; Revina, A.A. Synthesis of metallic nanoparticles in reverse micelles in the presence of quercetin. Colloids Surf. A Physicochem. Eng. Asp. 2000, 168, 87–96.

[68]. Khalil MMH, Soliman N, Abdel-Moaty HI, Ismael E, Sabry DY. Anti-Helicobacter Pylori, Anti-diabetic and Cytotoxicity Activity of Biosynthesized Gold Nanoparticles Using Moricandia nitens Water Extract. Egypt. J. Chem. 2018, 61(4), 691-703.

[69]. Otunola, G.A.; Afolayan, A.J.; Ajayi, E.O.; Odeyemi, S.W. Characterization, antibacterial and antioxidant properties of silver nanoparticles synthesized from aqueous extracts of *Allium sativum*, *Zingiber officinale*, and *Capsicum frutescens*. Pharmacogn. Mag. 2017, *13* (Suppl. 2), S201–S208. doi:10.4103/pm.pm_430_16.

[70]. Elsayed, M. M.; Elgohary, F. A.; Zakaria, A. I.; Elkenany, R. M.; EL-Khateeb, A. Y. Novel eradication methods for *Staphylococcus aureus* biofilm in poultry farms and abattoirs using disinfectants loaded with silver and copper nanoparticles. Environ. Sci. Pollut. Res. Int. 2020, 1-13. https://doi.org/10.1007/s11356-020-09340-9

[71]. Bhattacharjee, S. DLS and zeta potential—What they are and what they are not? J. Control. Release 2016, *235*, 337–351.

[72]. Honary, S.; Zahir, F. Effect of zeta potential on the properties of nano-drug delivery systems—A review (Part 2). Trop. J. Pharm. Res. 2013, 12, 265–273.

[73]. El-Shahaby, O.; El-Zayat, M.; Salih, E.; El-Sherbiny, I.M.; Reicha, F.M. Evaluation of antimicrobial activity of water infusion plant-mediated silver nanoparticles. Nanomed. Nanotechol. 2013, 4(2), 1–7.

[74]. Elazzouzi, H.; Zekri, N.; Zair, T.; El Belghiti, M.A. Volatiles profiling and antioxidant activity of Moroccan Artemisia ifranensis J. Didier and Anacyclus pyrethrum Link essential oils. Egypt. J. Chem. 2020, 63(10), 3937-3947.

[75]. Ibrahim, F. Y.; EL-Khateeb, A. Y.; Mohamed, A. H. Rhus and Safflower Extracts as Potential Novel Food Antioxidant, Anticancer, and Antimicrobial Agents Using Nanotechnology. Foods, 2019, 8, 139. https://doi.org/10.3390/foods8040139.

[76]. Kaileh, M.; Vanden Berghe, W.; Heyerick, A.; Horion, J.; Piette, J.; Libert, C.; De Keukeleire, D.; Essawi, T.; Haegeman, G. Withaferin a strongly elicits IkappaB kinase beta hyperphosphorylation concomitant with potent inhibition of its kinase activity. J. Biol. Chem. 2007, 282, 4253–4264.

[77]. Alfaifi, M.Y.; Saleh, K.A.; El-Boushnak, M.A.; Elbehairi, S.E.; Alshehri, M.A.; Shati, A.A. Antiproliferative Activity of the Methanolic Extract of Withania Somnifera Leaves from Faifa Mountains, Southwest Saudi Arabia, against Several Human Cancer Cell Lines. Asian Pac. J. Cancer Prev. 2016, 17, 2723–2726.

[78]. Shah, N.; Kataria, H.; Kaul, S.C.; Ishii, T.; Kaur, G.; Wadhwa, R. Effect of the alcoholic extract of Ashwagandha leaves and its components on proliferation, migration, and differentiation of glioblastoma cells: Combinational approach for enhanced differentiation. Cancer Sci. 2009, 100, 1740–1747.

[79]. Shah, N.; Singh, R.; Sarangi, U.; Saxena, N.; Chaudhary, A.; Kaur, G.; Kaul, S.C.; Wadhwa, R. Combinations of Ashwagandha leaf extracts protect brain-derived cells against oxidative stress and induce differentiation. PLoS ONE 2015, 10, e0120554.

[80]. Ahmed, W.; Mofed, D.; Zekri, A.R.; El-Sayed, N.; Rahouma, M.; Sabet, S. Antioxidant activity and apoptotic induction as mechanisms of action of *Withania somnifera* (Ashwagandha) against a hepatocellular carcinoma cell line. J. Int. Med. Res. 2018, 46, 1358–1369.

[81]. Wadhwa, R.; Singh, R.; Gao, R.; Shah, N.; Widodo, N.; Nakamoto, T.; Ishida, Y.; Terao, K.; Kaul, S.C. Water extract of Ashwagandha leaves has anticancer activity: Identification of an active component and its mechanism of action. PLoS ONE 2013, 8, e77189.

[82]. Kataria, H.; Kumar, S.; Chaudhary, H.; Kaur, G. *Withania somnifera* Suppresses Tumor Growth of Intracranial Allograft of Glioma Cells. Mol. Neurobiol. 2016, 53, 4143–4158.

[83]. Afifyorcid, M.; Samy, N.; Hafez, N A.; Alazzouni, A. S.; Mahdy, E.; El-Mezayen, H. A. E.; Kelany, M. M. Evaluation of Zinc-oxide Nanoparticles Effect on Treatment of Diabetes in Streptozotocin-induced Diabetic Rats. Egypt. J. Chem. 2019, 62 (10), 1771-1783. doi: 10.21608/ejchem.2019.11350.1735.

[84]. Abdel-Aziz, A.-W. A.; Abdel-Motagaly, M.; Abdallah, M. A.; Shaaban, R. S.; Osman, N. S.; Elwan, N. M. *In vitro* Cytotoxicity, Antimicrobial, Antioxidant Activities and HPLC Finger Print Analyses of the Extracts of Ceiba insignis Leaves Growing in Egypt. Egypt. J. Chem. Articles in Press, Accepted Manuscript, Available Online from 27 April 2021. doi: 10.21608/ejchem.2021.73023.3618

[85]. Banoon, S.R.; Ghasemian, A. The Characters of Graphene Oxide Nanoparticles and Doxorubicin Against HCT-116 Colorectal Cancer Cells *In Vitro*. J. Gastrointest. Canc. 2021. https://doi.org/10.1007/s12029-021-00625-x.

[86]. Saleh, A.; Elrefaie, H.; Hashem, A.; EL-Menoufy, H.; Mansour, N.; El-Beih, A. Optimization Studies and Chemical Investigations of Aspergillus terreus-18 Showing Antioxidant Activity. Egypt. J. Chem. 2019, 62(2), 215-230.